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Characterization of a Fatty Acid Synthetase from *Corynebacterium diphtheriae*

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SUMMARY

A fatty acid synthetase from *Corynebacterium diphtheriae* has been purified to a specific activity of 450 nmoles of malonyl coenzyme A incorporated per min per mg. The enzyme is optimally active in 0.5 M phosphate buffer. *C. diphtheriae* appears to be the most primitive organism having a multi-enzyme complex for fatty acid synthesis.

The fatty acid synthetases of animals (1-4), yeast (5), and other fungi (6) are multienzyme complexes which have been called type I synthetases (7). Type II synthetases, which require added acyl carrier protein for activity, have been observed in plants (8, 9) and bacteria (10, 11). Exceptions to this generalization have been reported. *Mycobacterium phlei*, one of the more advanced procaryotes, has a type I synthetase (7), and green *Euglena gracilis* appears to have both types of fatty acid synthetases (12).

Fatty acid synthesis in *Corynebacterium diphtheriae* was investigated to determine whether *M. phlei* was a singular example of a procaryote having a type I synthetase and whether phylogenetic relationships between organisms and the types of fatty acid synthetases exist.

EXPERIMENTAL PROCEDURE AND RESULTS

Enzyme Assay—Unless stated differently, the assay mixture had a volume of 0.5 ml and contained 250 μ moles of potassium phosphate (pH 7.3, optimum pH), 15 nmoles of TPNH, 15 nmoles of DPNH, 1 nmole of FMN, 50 nmoles of acetyl-CoA, 10 nmoles of [2-¹⁴C]malonyl-CoA (2 μ Ci per μ mole), 3 μ moles of dithiothreitol, 50 μ g of bovine serum albumin, and 0.11 μ g of enzyme protein. Activity, incorporation of radioactivity from [2-¹⁴C]malonyl-CoA into fatty acids, was determined as described previously (13).

Culture—A culture of *C. diphtheriae*, strain C-7, S(-), tox(-), was a gift from Dr. A. M. Papperheimer of the Biological Laboratories, Harvard University. The organisms, grown at 30° in a medium containing 10 g per liter of yeast extract and 15 g per

liter of maltose (65%, Tech grade) were harvested in the late log phase of growth.¹

Enzyme Purification¹—The washed cells were suspended in 0.55 M potassium-phosphate buffer, pH 8.0, which also contained 1 mM EDTA and 1 mM dithiothreitol, and were disrupted with a French Press. Centrifugation at 14,500 $\times g$ yielded a supernatant that was further purified. The purification procedures included (NH₄)₂SO₄ fractionation, centrifugation at 105,000 $\times g$, protamine sulfate precipitation of nucleic acids, hydroxyapatite adsorption, and DEAE-cellulose chromatography. A fraction from the chromatography procedure was diluted 1:20 in 0.55 M potassium buffer, pH 7.3, 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol. Aliquots of the diluted enzyme preparation were used for all assays unless indicated otherwise. The remainder of the pooled fractions was concentrated and stored at -70°.

The purification factor for the enzyme was 15.2-fold, and the preparation had a specific activity of 450 units per mg of protein (nanomoles of malonyl-CoA incorporated per min per mg) when assayed with bovine serum albumin, and 265 units per mg without bovine serum albumin. Higher specific activities (800 to 1000 units per mg) were observed in fractions obtained from high pressure chromatography of the enzyme preparation (see below), but such fractions rapidly deteriorated to reach specific activities of 300 to 400 units per mg.

Molecular Weight—The molecular weight, estimated by chromatography¹ with Sepharose 4B (Pharmacia), was found to be 2.5 and 3.0 $\times 10^6$ g per mole in two trials.

Products—The principal fatty acid synthesized was palmitic acid.¹ Bovine serum albumin and the polysaccharides from *M. phlei* that stimulate fatty acid synthesis alter the relative percentages of fatty acids synthesized (13).

Substrates and Cofactors—The stimulatory effect of bovine serum albumin was indicated above and has been investigated (13). Although the requirement for FMN was not absolute (62% activity when omitted), FAD was an ineffective substitute.

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¹ This paper represents a summary of a more complete manuscript that has been deposited as JBC Document No. 72M-1392. The deposited manuscript contains additional experimental data and procedural details relevant to the conclusions advanced in the paper. Order for supplementary material should specify the title, author(s), and reference to this paper and the JBC Document number, the form desired (microfiche or photocopy), and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014 and must be accompanied by remittance to the order of the Journal in the amount of \$2.50 per microfiche or \$4.05 per photocopy of 27 pages.

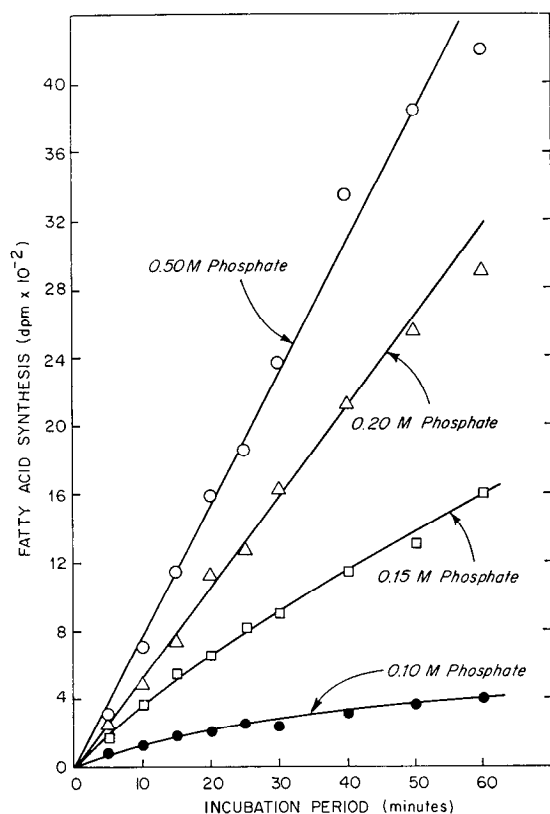


FIG. 1. The effect of various concentrations of phosphate on the fatty acid synthetase from *Corynebacterium diphtheriae*. Enzyme assays were those described in the text except that the phosphate buffer concentrations were varied as indicated.

Both DPNH and TPNH were required and higher concentrations of TPNH or the presence of DPN⁺ would not compensate for the omission of DPNH. The K_m values for substrates were: DPNH, 2.5 μ M; TPNH, 6 μ M; acetyl-CoA, 25 μ M; malonyl-CoA, 10 μ M.

The elongation of a series of fatty acyl-CoA derivatives, including propionic, isobutyric, and the even-numbered fatty acids with chain lengths between 4 and 16 were investigated. In all cases, activities were less than 10% of the value obtained with acetyl-CoA used as the substrate.

Effect of Phosphate—A relatively high concentration of a multivalent anion was required for enzyme activity. Phosphate, at 0.5 M, was the most effective of the anions tested, although citrate in lower concentrations was nearly equivalent to phosphate, and sulfate gave about 50% of the activity obtained with phosphate. The type of monovalent cation did not affect enzyme activity but Mg^{2+} caused precipitation in the assay mixture. The maximum activity for each salt appeared to be more directly correlated with the concentration of the anion, rather than the ionic strength of the assay medium. To determine whether the low enzyme activities observed for our enzyme in phosphate buffer of intermediate concentrations (0.1 to 0.2 M) were due to the inactivation of the enzyme, reaction rates, with various concentrations of phosphate buffer, were determined (Fig. 1). In further experiments, it was found that increasing the phosphate concentration (from 0.1 or 0.2 M to 0.5 M) at the midpoint of an incubation period increased fatty acid synthesis. Since the enzyme was in a 0.5 M phosphate solution prior to being added to the assay mixture, these results suggest that the catalytic efficiency of the

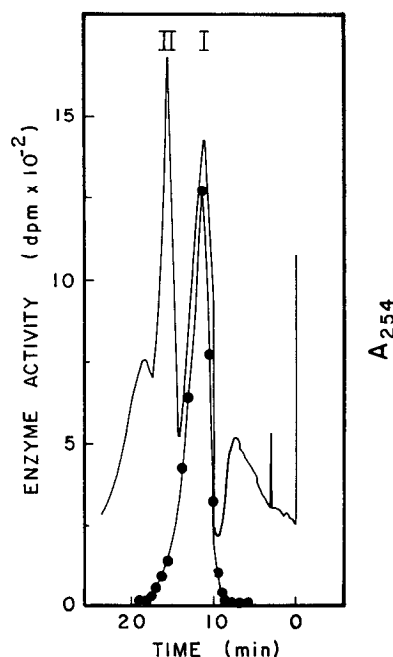


FIG. 2. High pressure chromatography of the fatty acid synthetase preparation from *Corynebacterium diphtheriae*. ●—●, enzyme activity (disintegrations per min $\times 10^{-2}$); —, ultraviolet absorbance. Enzyme assays and chromatography conditions are given in the text.

synthetase is rapidly and reversibly affected by phosphate concentrations in the range of 0.1 to 0.5 M.

When the enzyme was placed in 0.01 phosphate buffer at 37°, it was irreversibly inactivated within 5 min. At 0° the rate of inactivation was slower and was partially reversible. After 4 hours in 0.01 M phosphate buffer, at 0°, the enzyme had retained 10% of its original activity. When the phosphate concentration of this solution was raised to 0.5 M, enzyme activity increased, over a 20-hour period, to about 50% of that observed with an enzyme solution diluted to the same extent in 0.5 M phosphate and stored for 20 hours. A plot of log activity versus time¹ indicated that the inactivation process involved more than one reaction since there was a break between the linear portions of the plot.

High Pressure Chromatography—Procedures for high pressure, gel permeation chromatography were developed by Dr. I. Schechter.^{1,2} The stainless steel column (4 feet \times $\frac{3}{8}$ inch), packed with EM-Gel, type SI (Wolem) and the instrument equipped with a ultraviolet monitor was obtained from Chromatech, Inc. The column was treated with carbowax before used, and a 0.4 M potassium-phosphate buffer, pH 7.3 was the eluent. Conditions were: temperature, 9°; pressure, 300 p.s.i.; flow rate, 3 ml per min; fraction volume, 1.5 ml. A synthetase preparation, specific activity about 400 units per mg, was applied to the column.

As shown in Fig. 2, enzyme activity was present in one peak (I). Rechromatography of Peak I yielded both peaks (I and II) with about equal areas, but rechromatography of Peak II gave only Peak II. These results suggest that the molecules of Peak I fraction dissociate readily. To determine whether dissociation was involved in reversible inactivation of the enzyme in 0.01 M phosphate, enzyme was diluted into 0.01 M phosphate buffer at 0°. After 4 hours, the phosphate concentration was adjusted to 0.5 M in one-half of the solution and both samples were stored at

² Unpublished results, this laboratory.

0° for 16 more hours. High pressure chromatography revealed that, compared to Fig. 2, Peak II was much larger than Peak I in both samples, but the ratios (I:II) of the peak areas (protein) for the inactivated (less than 10% of original activity) and reactivated (40% of original activity) samples were not significantly different.¹

Thus the lowering of phosphate concentration to 0.01 M results in (a) a reversible inactivation without dissociation of the complex, and (b) a dissociation that is irreversible under the conditions used. These two processes seem to be distinct from the effects seen in the range of 0.1 to 0.5 M phosphate.

DISCUSSION

Comparing the properties of the *C. diphtheriae*, *M. phlei*, and yeast enzymes, it appears that the effect of phosphate concentration in the range of 0.1 to 0.5 M is distinctive for the *C. diphtheriae* synthetase. The enzyme from *C. diphtheriae* does resemble the *M. phlei* synthetase in that both lack stability in buffers of low ionic strength (7) and that DPNH as well as TPNH are required electron donors (14). However, the K_m values for TPNH and for malonyl-CoA are similar for the synthetases from all three sources (5, 14, 15). The *C. diphtheriae* enzyme resembles the yeast enzyme with respect to molecular weight, products, K_m for acetyl-CoA, and the response of the enzymes to bovine serum albumin and to the stimulatory polysaccharides from *M. phlei* (5, 7, 13). Thus a direct correlation of enzyme properties to supposed evolutionary development is not apparent when these three enzymes are considered; on the other hand, the presence of type I fatty acid synthetases in *C. diphtheriae*, *M. phlei*, and

*Streptomyces coelicolor*³ indicates that the multienzyme complex form may be common among the more advanced bacteria.

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³ M. Weinrich, unpublished results, this laboratory.